AD_____

Award Number: DAMD17-97-1-7236

TITLE: Identification of Tumor Suppressor Genes in Breast Cancer by International Mutagenesis and Functional Inactivation

PRINCIPAL INVESTIGATOR: Yan An. Su, M.D., Ph.D.

CONTRACTING ORGANIZATION: National Institute of Health
Bethesda, Maryland 20892-4470

REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual (25 Aug 99 - 25 Aug 00)

4. TITLE AND SUBTITLE
Identification of Tumor Suppressor Genes in Breast Cancer by International Mutagenesis and Functional Inactivation

5. FUNDING NUMBERS
DAMD17-97-1-7236

6. AUTHOR(S)
Yan An. Su, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
National Institute of Health
Bethesda, Maryland 20892-4470

E-MAIL:
suya@gunet.goergetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The identification of tumor suppressor genes inactivated and proto-oncogenes activated in mammary epithelial cells is essential to understand the genetic basis of breast cancer and is a prerequisite for development of strategies for prevention, diagnosis, and treatment. We are applying the novel retroviral-tagging strategy to identify genomic loci and genes involved in breast cancer progression using chromosome 17-mediated suppressed (independent of p53 and BRCA1) breast cancer cell line CAL/17. In contrast to the parental tumorigenic cell line CAL51, the suppressed subline CAL/17 display insulin-dependent growth in plastic culture, anchorage-dependent growth in soft-agar culture and no tumor formation in athymic nude mice. We now report the successful selection and identification of anchorage-independent cell sublines and insulin-independent cell sublines induced by retrovirus-insertional mutagenesis of the CAL/17 cell line. The resultant 5 anchorage-independent cell sublines and 10 insulin-independent cell sublines were used to clone the genomic sequences at the retroviral insertion sites. 5 retroviral insertion sites have been mapped to chromosome regions including chromosome 7q11-q21, chromosome 14, chromosome 15q26.1, and chromosome 22. We are in the process to complete the chromosome mapping of retroviral vectors in the remaining 10 revertant cell sublines and to conduct tumorigenicity tests in athymic nude mice.

14. SUBJECT TERMS
Breast cancer, Chromosome alteration and transfer, Tumor Suppression, Insertional mutagenesis, Anchorage-dependent Anchorage-independent, Insulin-dependent, and Insulin-independent

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Breast cancer is the most common malignancy in Western women, affecting up to one in 10 women during their lifetime and approximately 40,000 women dying from the disease each year in the U.S. Tumor genetic profiling through such methods as loss of heterozygosity (LOH) screening, comparative genomic hybridization (CGH), and cDNA microarrays all point to the same conclusion that a number of genetic changes are responsible for the malignant phenotype. For example, genes involved in breast cancer progression include amplification of oncogenes such as MYC, ERBB2, CCND1 and mutation of tumor suppressor genes TP53 and CHD1[1]. In case of hereditary breast cancer, germline mutations of tumor suppressor genes PTEN on chromosome 10q23.3, ATM on chromosome 11q22-q23, BRCA1 on chromosome 17q21, and BRCA2 on chromosome 13q12.3 were also shown to involve in the tumor progression [2]. These data represent a significant advance in our understanding of molecular genetics of breast cancer. However, breast cancer is a heterogeneous disease that entails complex genetic alterations in which tumor suppressor genes, oncogenes, and modulator genes were mutated. Multistep genetic alterations transform normal mammary epithelial cells via the steps of hyperplasia, premalignant change, in situ carcinoma, invasion, and metastases. Genome-wide searching for the alterations and the elucidation of molecular events involved in these steps is the main focus for new strategies targeted at diagnosis, prevention and treatment.

While many investigators have observed LOH in breast cancers on chromosomes 1p, 1q, 3p, 6q, 7q, 11p, 13q, 16q, 17p, 17q, and 18q at the high frequencies variable from 20-60%, chromosome 17 is one of the most frequent carriers of LOH [3-15]. On 17p, two distinct regions, 17p13.1 (containing *p53*) and 17p13.3, have shown LOH with frequencies ranging from 30-60% and 60-70%, respectively [10;11;16-22]. On 17q, three regions of frequent LOH have been identified. 17q21 LOH [12;22-25] contains *BRCA1* [26]. LOH at 17q11.1-q12 was detected as frequently as 79% in sporadic breast cancer [27]. The third LOH is telomeric to *BRCA1* [22;24]. These studies demonstrate that, in addition to *p53*, *BRCA1, BRCA2*, and others, chromosome 17 and other chromosomes with the high frequent areas of LOH harbor unrecognized tumor suppressor genes involved in the control of the normal growth of mammary epithelial cells.

Direct evidence supporting the existence of additional breast cancer suppressor genes comes from introduction of a *neo*-tagged chromosome 17 into breast cancer cell lines by microcell-mediated chromosome transfer that demonstrated suppression of tumorigenicity [28-32]. Casey *et al.* was the first to provide biological evidence that *in vitro* growth of the breast cancer cell line MCF 7 (carrying wild-type *p53*) was suppressed by the introduction of a *neo*-tagged chromosome 17 [28]. A very similar result was independently reported by Negrini *et al.* [29]. In addition, anchorage-independent growth, cell growth rate on plastic plates, and tumorigenicity in athymic nude mice of the mammary carcinoma cell line R30 were suppressed by introduction of chromosome 17. Wild-type *p53* was not involved in this suppression [30]. Furthermore, only the long arm of the transferred chromosome 17 was capable of suppressing the tumorigenicity of the *p53*-mutant breast cancer cell line MDA-MD-231 [29]. Finally, Theile *et al.* demonstrated that suppression of tumorigenicity of the breast cancer cell line CAL51 by an introduced chromosome 17 did not require transfer of *p53* or *BRCA1* [32]. Thus, additional tumor suppressor genes on chromosome 17 have yet to be identified.

The insulin growth factor family was shown to play a role in breast cancer growth and progression. In fact, hyperinsulinemia, resulting from diet, aging, obesity and inadequate exercise, is a risk factor for breast cancer. It is also associated with the early stages of breast cancer [33] [34]. The insulin growth factor family is comprised of two ligands insulin-like growth factor I (IGF1) and insulin-like growth factor II (IGFII), three receptors (IGFIR, IGFIIR and insulin receptor) and seven binding proteins (IGFBP1-7). Both IGFI and IGFII activate estrogen receptor (ER)-dependent transcription in the presence of liganded ER. Conversely, estrogen regulates the expression of IGFs, IGFIR and IGFBPs [35] [36] [37]. In addition, IGFIR levels are a key modulator of cellular estrogen sensitivity [38]. Further elucidation of relationship between insulin-like growth factor superfamily and estrogen resposiveness involved in breast cancer growth should facilitate our understanding of the malignancy.

To facilitate identification of tumor suppressor genes we have developed a novel strategy to reverse monochromosome-mediated tumor suppression by retroviral insertional mutagenesis and/or functional inactivation mediated by expressed cDNA fragments [39]. Insertional mutagenesis disrupts tumor suppressor genes (e.g., *APC* for familial adenomatous polyposis [40] and *p53* in osteosarcoma [41]) and has been used as a powerful tool to identify various genes including *Fli-1*, *p53*, *erb-B*, and *myc* [42-44], *vin-1/cyclin D2* [45], *Tiam-1* [46], *bcar-1* [47], and *CRL-1* [48]. Furthermore, it is known that retroviral insertion could activate proto-oncogene. Using retroviral insertional mutagenesis, we have developed a novel strategy for identification of genetic loci and tumor suppressor genes [39].

"Suppression and reversion of suppression" are the two basic aspects of this novel strategy. "Suppression" refers to the suppression of tumorigenic phenotypic features including anchorage-independent growth, focus formation in plastic culture, rapid cell population doubling time, and tumor formation in athymic nude mice by introduction of a *neo*-tagged monochromosome into a cancer cell line via a microcell mediated chromosome transfer. "Reversion of suppression" means the reversion to the tumorigenic phenotype induced by insertional mutagenesis (proviral tagging) and/or functional inactivation of the suppressor gene(s) by antisense or dominant negative mutant proteins following the transduction of a retrovirus expression vector-carried cDNA library into the monochromosome suppressed cells.

The hypothesis underlying this approach is the following. (1) The phenotypic reversion can derive from inactivation of tumor suppressor genes. The suppression related genetic locus or loci on the introduced chromosome provide targets to insertional mutagenesis. In addition, derived from cancer cell lines, the suppressed sublines may carry many mutated genetic loci that leave functional counterparts being "haploid" targets to insertional mutagenesis. Furthermore, the phenotypic reversion can come from activation of proto-oncogenes. (2) The successfully transduced tumorigenic cells can be positively selected in soft agar culture following co-selection for the drug-resistance genes on both the suppressive chromosome and the retroviral vector. (3) The genomic sequences tagged to the vectors and involved in the tumorigenic reversion can be readily isolated by PCR-based techniques. Based on this hypothesis we have successfully generated a serial retroviral-tagged revertant cell sublines using the chromosome-6 suppressed melanoma cell line UACC903(+6) [39].

We are applying the same strategy to identify unrecognized tumor suppressor gene(s) from a chromosome-17 suppressed breast cancer cell sublines. This study is using the tumorigenic cell line CAL51 and the chromosome-17 suppressed cell subline CAL/17 [32]. The parental CAL51 cell line demonstrates insulin-independent growth, anchorage-independent growth, and rapid formation of subcutaneous tumors in athymic nude mice. All these readily detectable phenotypic

features are suppressed in the chromosome 17 containing cell subline CAL/17 [32]. The central goal of this project is to identify breast cancer suppressor genes. The specific aims include (1) use of the chromosome-17 suppressed breast cancer cell sublines CAL/17 to generate the anchorage-independent revertants and (2) use of the anchorage-independent revertants to identify previously unrecognized suppressor genes. We previously reported the successful selection of the anchorage-independent cell sublines and the insulin-independent cell sublines. In this report, we describe (1) establishment of 5 stable anchorage-independent cell sublines and 10 stable insulin-independent cell sublines induced from the CAL/17 cell line by the retroviral insertional mutagenesis, (2) development of a polymerase chain reaction (PCR)-based method for rapid cloning of genomic sequences at the retroviral integration sites, (3) nucleotide sequence analysis, and (4) chromosomal localization of cloned genomic sequences.

(6) BODY

Below are the timetable and the technique objectives from our original proposal. The current status of the technique objectives is indicated in the parentheses. The detailed description follows the Statement of Work.

Table 1. The Timetable for the Proposed Experiments

	Specific Aim 1	Specific Aim 2
Year 01		
	- Completion of the library construction	
	- Completion of the retrovirus package	
	- Completion of the retrovirus transduction	
Year 02		
	- Completion of soft agar selection for colony forming cells and establishment of revertant sublines	
		- Study of *in vitro* growth and tumorigenicity test
		- Identification of the effective cDNAs
Year 03		
		- Mapping of identified cDNAs to chromosome regions
		- Characterization of the full length cDNAs

Technique Objectives

Task 1: Months 1-2: Cell culture of the CAL51 and CAL/17-1 cells. Isolation of poly(A)$^+$RNA from the chromosome-17 suppressed cell line CAL/17-1 for construction of a cDNA library. Isolation of DNA and total RNA from the two cell sublines for the future Southern and Northern analysis **(Completed)**.

Task 2: Months 2-3: Transfection of pLM2 plasmid vector into CAL51 and CAL/17-1 cells to determine the killing curves in the presence of L-histidinol dihydrochloride **(Completed)**.

Task 3: Months 3-4: Soft agar culture of the L-histidinol-resistant CAL51 and CAL/17-1 sublines (transfected with pLM2 vector) to generate the first-hand data for soft agar selection experiments **(Completed)**.

Task 4: Months 3-5: Construction of a random primed normalized cDNA library onto pLM2 vectors using poly(A)$^+$RNA isolated from CAL/17-1 cells **(Modified and completed)**.

Task 5: Months 6-8: Package of the ecotropic and amphotropic retrovirus particles from the pLM2-carried cDNA library and determination of a titer of the retrovirus particles. **(Completed)**.

Task 6: Months 9-11: Transduction of the retrovirus particles into the CAL/17-1 cells and selection for colony forming cells using soft agar culture. **(Completed)**.

Task 7: Months 11-14: Individual colonies will be lifted from soft agar culture. Cells from the individual colonies will be expanded on plastic culture and re-plated in soft agar culture to eliminate false positives and to establish true revertant sublines **(Completed)**.

Task 8: Months 14-16: Study of *in vitro* growth of anchorage-independent revertant cell sublines to select candidates for test of tumorigenicity in athymic nude mice **(Completed)**.

5 stable anchorage-independent revertant cell sublines were generated. In addition, 10 stable insulin-independent revertant cell sublines were also generated. Manuscripts for reversion and selection of anchorage-independent cell sublines and insulin-independent cell sublines are in preparation.

Task 9: Months 17-20: Tumorigenicity tests of the candidate sublines in athymic nude mice to identify the tumorigenic sublines **(In progress)**.

Task 10: Months 19-23: Identification of cDNA inserts on the integrated retroviral vectors. Test of effects of identified cDNAs on tumorigenic reversion of the CAL/17-1 cell line. **(Was modified)**.

Task 11: Months 21-24: Cloning of genomic sequences flanking the integrated retrovirus vectors **(Completed)** and subsequent identification of their encoded or adjacent cDNAs **(In progress)**.

A PCR-based method for rapid cloning genomic sequences at the retroviral integration sites was developed, and a manuscript is in preparation.

Task 12: Months 25-27: Localization of identified cDNAs and flanking sequences onto chromosome regions **(Completed in part)**.

Task 13: Months 28-36: Molecular characterization of newly identified breast cancer suppressor gene(s) **(To be done)**.

MATERIALS, METHODS, AND PROCEDURES

Cell Culture. The parental breast cancer cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17 [32] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 ug/mL streptomycin sulfate. Six hundred ug/mL of G418 and 10 ug/mL insulin were added in culture of the chromosome 17-containing cells. The insulin-independent revertant cell sublines are cultured in the absence of the insulin. Bosc23 cells [49] and Bing [50] cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 ug/mL streptomycin sulfate. Ten% of newborn calf serum was used for culture of GP+envAM12 cells [51]. Eight mM of L-histidinol dihydrochloride (*his*D^R) was used for cells containing retrovirus vectors. All media, serum, and antibiotics were from Gibco BRL with exceptions where indicated.

Escherichia coli strain DH5α cells (GIBCO, BRL) containing plasmid pLM2 [39] and strain Supercompetent cells (Catalogue no. 230140; Stratagene) containing a cDNA library were cultured in Luria-Bertani medium with 100 ug of ampicillin per ml. *E. coli* DH5α (GIBCO,

BRL) carrying pAMP10 plasmid and its derivatives were cultured in Luria-Bertani (LB) medium with 100 ug/ml ampicillin. LB agar medium with 100 μg/ml ampicillin, 40 μg of isopropyl-β-thiogalactopyranoside (IPTG) and 40 μg of 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-gal) per ml (both from Sigma) was used to detect insertion into pAMP10.

Techniques of the Molecular Cloning. Genomic DNA were isolated by standard methods [52]. Poly(A)$^+$-RNA was extracted by use of the FastTrack 2.0 mRNA Isolation Kit according to manufacturer's instructions (Catalog nos. K1593-02, K1593-03; Invitrogen). The cDNA library was synthesized from poly(A)$^+$ RNA using Universal RiboClone cDNA Synthesis System (Catalog no. C4360; Promega) according to the manufacturer's instruction. Briefly, first strand synthesis was driven by Avian Myeloblstosis Virus (AMV) reverse transcriptase and random hexameric primers, followed directly by second strand replacement synthesis using Rnase H and DNA polymerase I. After treatment with T4 DNA polymerase to flush the ends, the double-stranded cDNA molecules were prepared for cloning by size fractionation and the additino of *Eco*RI adaptors. The resulting cDNA samples were cloned into pLM2 plasmid vectors. To generate high transformation efficiency, the double stranded cDNA were phosphorylated and pLM2 vectors were dephosphorylated before ligation. Plasmid DNA was isolated using alkaline lysis methods [53] or using QIAGEN Plasmid Purification Kit (catalogue No. 12262) according to the manufacturer's instruction.

Packaging of Retrovirus Particles. Virus particles containing retroviral vectors were packaged from pLM2 plasmids, using the retrovirus-packaging cell lines Bosc-23 (ecotropic) and GP+envAM12 (amphotropic) and BING (amphotropic) by methods essentially as described [51] [49;50]. Briefly, 50 ug of plasmid DNA or pLM2-carried cDNA library were used to transfect approximately 10^7 Bosc-23 or BING cells using Cell-Porator Electroporation System I (catalogue No. 71600-019; GIBCO BRL) according to the manufacturer's instruction. To determine the transfection efficiency, 50-ug plasmid vectors with a green fluorescence gene was transfected into the same cells under the same conditions. 10 ml of supernatant from transfected Bosc-23 cells were also used to transduce aliquots of 1 x 10^6 GP+envAM12 cells in the presence of 6 ug Polybrene per ml for production of the amphotropic virus particles. The pLM2-virus vector-containing cells are selected for with with L-histidinol. The amphotropic virus particles from packaged from the Bing cells were used to transduce the breast cancer cell lines.

Transduction. 15 T75-flasks of the chromosome 17-mediated suppressed cells (CAL/17-1) were cultured up to 50% confluence (approximately 10^7 cells/flask) and transduced with amphotropic virus particles in the transduction medium. The aliquots of 10 ml transduction medium consist of 5 ml culture medium and 5 ml supernatant from the transduced BING cells. 4-ug polybrene were added in each ml medium to enhance the attachment of the virus particles to the cell surface. The fresh transduction medium was used to replace the old one every 4 hours per day for three days. The transduced cells were selected for with both G418 and HisD for 10 days.

Soft agar selection. Approximately 3 x 10^6 transduced cells were cultured in 0.33% soft agarose based on 0.9% bottom agarose in the concentration of 50,000 per 60-mm dish. After 4-week culture, all the cells from the top agarose were combined for the second round of the soft agar selection to enrich the true positive anchorage-independent cells. After the second round

selection, 2 clones of colony forming cells were lifted from each 35-mm dish and a total of 96 clones were selected for establishment of permanent anchorage-independent revertant cell sublines.

Selection for insulin-independent cell sublines. Approximately 5 x 10^6 transduced cells were cultured in the medium without insulin for two months to select for insulin-independent cell sublines. The CAL51 and CAL/17 cells were used as the positive and negative controls, respectively. 48 clones were selected for establishment of permanent insulin-independent revertant cell sublines.

Southern Blot Hybridization. Genomic DNA (20 ug) was digested with restriction enzymes *Hin*dIII (Catalogue No. 104CS, New England BioLabs Inc.) and *Eco*RI (Catalogue No. 101CS, New England BioLabs Inc.). The digested DNA were size-fractionated on 0.8% agarose gel. The DNA in the gel were denatured and neutralized, and then blotted to Hybond-N^+ nylon membrane (Catalogue No. RPN. 303N Amersham Pharmacia Biotech) by capillary transfer. The DNA in the membrane was UV cross-linked (UV Strantalinker 1800, Stratagene, La Jolla, CA). A *His*D DNA probe was prepared by *Bam*HI-digestion and purified from a gel using the GENECLEAN II Kit (Bio 101, Inc.) and labeled with [α^{32}p]-dCTP using the Random Primed DNA Labeling Kit (Boehringer Mannheim). The membrane was prehybridized at 65° C for 3 hours in a prehybridization solution (5×Denhardt's, 5×SSPE, 0.5% SDS) and then hybridized with the [α^{32}p]-dCTP labeled *His*D probe in the same solution at 65° C overnight. Washes were performed in 1×SSPE/0.1% SDS at room temperature for 10 min and twice with 0.1×SSPE/0.1%SDS at 42° C for 15 min. The membranes were exposed to autoradiogram films (Catalogue No. Labscientific Inc., Livington N.J. 07039) at -80° C in the presence of intensifying screens for 5-7 days.

Cloning of Genomic Sequence at Retrovirus integration site. Single-stranded DNA was amplified by the vector specific primer (SN158, 5'-TCGGTGGTCCCTGGGCAGGGGTCTCCAAAT-3') from the 5'-end of the retrovirus vector. PCR was performed in 50 µl reaction mixtures made up of 0.5 µg of template genomic DNA, 0.2 µM of oligonucleotide primer, 100 µM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 1×PCR buffer, and 5 units Taq polymerase. The reaction was heated to 94° C for 5 min, and 35 cycles of 94° C for 1 min, 56° C for 2 min and 72° C for 1 min, followed by final extension for 10 min at 72° C. The PCR products were purified with Micro Bio-Spin 30 chromatography columns (Catalogue No. 732-6223, BIO-RAD) as recommended by the manufacturer.

The tailing reaction contained 15 µL (0.5 µg) of the purified amplication PCR products, 100 nM of deoxynucleotide triphosphate (Catalogue No. 27-2035-01; Amersham Pharmacia Biotech), 20 U of terminal deoxynucleotidyl transferase (Catalogue No. 252S, New England BioLabs Inc.), 1× reaction buffer for terminal transferase containing 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT and 0.25 mM $CoCl_2$ in a final volume of 50 µL. The reaction mixture was incubated at 37° C for 1 hour and then heat to 70° C for 10 minutes to inactivate the enzyme.

The poly(dA)-tailed PCR products were used as templates for secondary amplification by PCR with nested primers. The 50 µl reaction contained 0.2 µM nested vector-specific primer (SN121: 5'-TTGTTCCTGACCTTGATCTGAACT-3'), 0.2 µM poly(dT)-primer (5'-

CGGAGGTTTTTTTTTTTTTTTT-3'), and 2 μL of the poly(dA)-tailed fragments. The other components were the same as those for the single-stranded PCR. The reaction comprised one cycle at 94° for 5 min, and 25 cycles of denaturation for 1 min at 94° C, annealing for 2 min at 48° C and extension for 1 min at 72° C followed by final extension for 10 min at 72° C. The third PCR was performed with 0.2 μM primers SN127 (5'-CUACUACUACUATCCATGCCTTGCAAAGTGGCGTTA-3') and SN81 (5'-CUACUACUACUACGGAGGTTTTTTTTTTTTTTTT-3']. Both primers contained $(CUA)_4$ sequence at the 5'-end for cloning of the PCR products to the UDG-cloning vector pAMP10 [54]. The PCR reaction was the same as the secondary PCR reaction with the exception of the annealing temperature of 50° C. The PCR products were purified using Micro Bio-Spin 30 chromatography columns.

50 ng of PCR products were annealed with 25 ng of pAMP10 plasmid vectors (Catalogue No. 18552-018, Life Technologies Inc.) as recommended by the supplier. The recombinant plasmids were transformed into competent *E. coli* DH5α cells for screening white colonies that contain DNA inserts. All the white colonies were picked up and transferred onto two duplication plates. After overnight culture at 37° C, the colonies were transferred to Hybond-N^+ nylon membranes (Catalogue No. RPN. 82C Amersham Pharmacia Biotech) for colony hybridization under the same conditions as Southern hybridization with the [γ^{32}p]dATP-labeled vector-specific probe SN212 (5'-AAGCTAGCTTGCCACCTACGGGTGGGGTCTTTCAAA-3'). Washes were performed in 1×SSPE/0.1% SDS at room temperature for 10 min and twice with 0.1×SSPE/0.1%SDS at 42 C for 15 min. The positive bacterial colonies were picked up for cell expansion. The plasmid DNA was isolated from dozens of colonies by utilization of a mini preparation kit (Catalogue No. 2072-400, Bio 101 Inc.) for nucleotide sequence analysis.

DNA sequence analysis. The insert sequences in the plasmid vectors pAMP10 were determined by use of a Perkin Elmer Applied Biosystem (PE-ABI) automated 377 DNA sequencer. The dye terminator (4 fluorescent dyes) labeling method and AppliTaq FS DNA polymerase were used for the DNA sequencing reaction. The sequencing reaction and gel electrophoresis were conducted following the manufacturer's instructions (PE-ABI). Multiple sequence alignments and consensus determinations were performed with DNASTAR software version 1.58 (DNASTAR Inc., Madison, WI). The sequence database GeneBank was searched by use of the BLASTN and BLASTX programs [55;56;56].

(7) KEY RESEARCH ACCOMPLISHMENT

This research has generated the following key research accomplishment.

a. 96 anchorage-independent revertant cell sublines were induced by retroviral insertional mutagenesis. 5 stable and unique anchorage-independent revertant cell sublines have been established.

b. 48 insulin-independent revertant cell sublines were induced by retroviral insertional mutagenesis. 10 stable and unique insulin-independent revertant cell sublines have been established.

c. A method for rapid cloning genomic sequences at the retroviral integration sites has been successfully developed. Using this method, we have cloned the genomic sequences at the retroviral insertion sites in all of the 5 anchorage-independent cell sublines and the 10 insulin-independent cell sublines.

d. 31 plasmid DNA were isolated from cloning of genomic sequences at the retroviral insertion sites in anchorage-independent cell sublines. 50 plasmid DNA were isolated from cloning of genomic sequences at the retroviral insertion sites in insulin-independent cell sublines. On average, 500 base pair (bp) sequences were cloned from each revertant cell subline.

e. Out of the 5 unique revertant anchorage-independent cell sublines, 2 integrated retroviral vectors were localized at chromosome 7q11-q21 and 15q26.1 band regions. The retroviral vectors in 3 revertant cell subline have yet to be mapped.

f. Out of the 10 unique revertant insulin-independent cell sublines, 3 integrated retroviral vectors were localized onto human chromosomes, one on chromosome 7q11-q21, one on chromosome 14, and one on chromosome 22. The retroviral vectors in 7 revertant cell subline have yet to be mapped.

(8) REPORTABLE OUTCOMES

The following manuscripts are in preparation for publication.

a. Title: "Reversion of the chromosome 17-mediated suppressed breast cancer cell line CAL/17 to anchorage-independent growth by retroviral insertional mutagenesis";

b. Title: "Reversion of the chromosome 17-mediated suppressed breast cancer cell line CAL/17 to insulin-independent growth by retroviral insertional mutagenesis";

c. Title: "Rapid cloning of genomic sequence at a retroviral insertion site";

(9) CONCLUSIONS

a. We have applied retroviral insertional mutagenesis to successfully revert the chromosome 17-mediated suppressed breast cancer phenotypic features including anchorage-dependent growth and insulin-dependent growth.

b. The resulting 5 anchorage-independent revertant breast cancer cell sublines are unique and useful for identification of genomic loci and genes involved in breast cancer progression from anchorage-dependent growth to anchorage-independent growth.

c. The resulting 10 insulin-independent revertant breast cancer cell sublines are also unique and provides innovative cellular resource for identification of genomic loci and genes involved in the insulin-dependent and insulin-independent growth.

d. The PCR-based method for rapid cloning of genomic sequence at a retroviral insertion site should be useful to clone any unknown nucleotide sequence adjacent to a known sequence.

e. The genomic loci identified at human chromosome 7q11-q21, 14, 15q26.1, and 22 should facilitate the identification of genes involved in the reversion of the suppressed phenotype to anchorage-independent growth or insulin-independent growth.

f. The determination of 10 other retroviral insertion sites in the 3 anchorage-independent revertant cell subline and 7 insulin-independent revertant cell lines are in progress. Completion of these mapping should result in the identification of more genomic loci and genes involved in the breast cancer progression.

g. The fact of our successful creation of the anchorage-independent and the insulin-independent cell sublines strongly suggest that the retroviral insertional mutagenesis can be applied to many areas of cancer research. For example, we can select for the hormone-independent cell sublines from a hormone-independent cell line in culture, and the tumorigenic cell sublines from a non-tumorigenic cell line and the metastatic cancer cell sublines from a non-metastatic cell line in animal models.

(10) REFERENCES

1. Ingvarsson S. Molecular genetics of breast cancer progression. Semin Cancer Biol 1999; 9: 277-288.
2. Tonin PN. Genes implicated in hereditary breast cancer syndromes. Semin Surg Oncol 2000; 18: 281-286.
3. Chen LC, Dollbaum C, Smith HS. Loss of heterozygosity on chromosome 1q in human breast cancer. Proc Natl Acad Sci U S A 1989; 86: 7204-7207.
4. Devilee P, van den BM, Kuipers-Dijkshoorn N et al. At least four different chromosomal regions are involved in loss of heterozygosity in human breast carcinoma. Genomics 1989; 5: 554-560.
5. Devilee P, van Vliet M, van Sloun P et al. Allelotype of human breast carcinoma: a second major site for loss of heterozygosity is on chromosome 6q. Oncogene 1991; 6: 1705-1711.
6. Bieche I, Champeme MH, Matifas F, Hacene K, Callahan R, Lidereau R. Loss of heterozygosity on chromosome 7q and aggressive primary breast cancer [see comments]. Lancet 1992; 339: 139-143.
7. Ali IU, Lidereau R, Theillet C, Callahan R. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science 1987; 238: 185-188.
8. Lundberg C, Skoog L, Cavenee WK, Nordenskjold M. Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. Proc Natl Acad Sci U S A 1987; 84: 2372-2376.
9. Radford DM, Fair KL, Phillips NJ et al. Allelotyping of ductal carcinoma in situ of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. Cancer Res 1995; 55: 3399-3405.
10. Mackay J, Steel CM, Elder PA, Forrest AP, Evans HJ. Allele loss on short arm of chromosome 17 in breast cancers. Lancet 1988; 2: 1384-1385.
11. Coles C, Thompson AM, Elder PA et al. Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. Lancet 1990; 336: 761-763.
12. Hall JM, Lee MK, Newman B et al. Linkage of early-onset familial breast cancer to chromosome 17q21. Science 1990; 250: 1684-1689.
13. Devilee P, van Vliet M, Kuipers-Dijkshoorn N, Pearson PL, Cornelisse CJ. Somatic genetic changes on chromosome 18 in breast carcinomas: is the DCC gene involved? Oncogene 1991; 6: 311-315.
14. Dutrillaux B, Gerbault-Seureau M, Zafrani B. Characterization of chromosomal anomalies in human breast cancer. A comparison of 30 paradiploid cases with few chromosome changes. Cancer Genet Cytogenet 1990; 49: 203-217.
15. Hainsworth PJ, Raphael KL, Stillwell RG, Bennett RC, Garson OM. Cytogenetic features of twenty-six primary breast cancers. Cancer Genet Cytogenet 1991; 53: 205-218.

16. Chen LC, Neubauer A, Kurisu W et al. Loss of heterozygosity on the short arm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer. Proc Natl Acad Sci U S A 1991; 88: 3847-3851.

17. Deng G, Chen LC, Schott DR et al. Loss of heterozygosity and p53 gene mutations in breast cancer. Cancer Res 1994; 54: 499-505.

18. Cornelis RS, van Vliet M, Vos CB et al. Evidence for a gene on 17p13.3, distal to TP53, as a target for allele loss in breast tumors without p53 mutations. Cancer Res 1994; 54: 4200-4206.

19. Sato T, Tanigami A, Yamakawa K et al. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 1990; 50: 7184-7189.

20. Andersen TI, Gaustad A, Ottestad L et al. Genetic alterations of the tumour suppressor gene regions 3p, 11p, 13q, 17p, and 17q in human breast carcinomas. Genes Chromosomes Cancer 1992; 4: 113-121.

21. Isomura M, Tanigami A, Saito H et al. Detailed analysis of loss of heterozygosity on chromosome band 17p13 in breast carcinoma on the basis of a high-resolution physical map with 29 markers. Genes Chromosomes Cancer 1994; 9: 173-179.

22. Kirchweger R, Zeillinger R, Schneeberger C, Speiser P, Louason G, Theillet C. Patterns of allele losses suggest the existence of five distinct regions of LOH on chromosome 17 in breast cancer. Int J Cancer 1994; 56: 193-199.

23. Cornelis RS, Devilee P, van Vliet M et al. Allele loss patterns on chromosome 17q in 109 breast carcinomas indicate at least two distinct target regions. Oncogene 1993; 8: 781-785.

24. Cropp CS, Champeme MH, Lidereau R, Callahan R. Identification of three regions on chromosome 17q in primary human breast carcinomas which are frequently deleted. Cancer Res 1993; 53: 5617-5619.

25. Borg A, Zhang QX, Johannsson O, Olsson H. High frequency of allelic imbalance at the BRCA1 region on chromosome 17q in both familial and sporadic ductal breast carcinomas. J Natl Cancer Inst 1994; 86: 792-794.

26. Miki Y, Swensen J, Shattuck-Eidens D et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 1994; 266: 66-71.

27. Futreal PA, Soderkvist P, Marks JR et al. Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. Cancer Res 1992; 52: 2624-2627.

28. Casey G, Plummer S, Hoeltge G, Scanlon D, Fasching C, Stanbridge EJ. Functional evidence for a breast cancer growth suppressor gene on chromosome 17. Hum Mol Genet 1993; 2: 1921-1927.

29. Negrini M, Sabbioni S, Haldar S et al. Tumor and growth suppression of breast cancer cells by chromosome 17- associated functions. Cancer Res 1994; 54: 1818-1824.

30. Theile M, Hartmann S, Naundorf H et al. Wild-type p53 is not involved in reversion of the tumorigenic phenotype of breast cancer cells after transfer of normal chromosome 17. Int J Oncol 1994; 4: 1067-1075.

31. Chen P, Ellmore N, Weissman BE. Functional evidence for a second tumor suppressor gene on human chromosome 17. Mol Cell Biol 1994; 14: 534-542.

32. Theile M, Hartmann S, Scherthan H et al. Suppression of tumorigenicity of breast cancer cells by transfer of human chromosome 17 does not require transferred BRCA1 and p53 genes. Oncogene 1995; 10: 439-447.

33. Stoll BA. Breast cancer: further metabolic-endocrine risk markers? Br J Cancer 1997; 76: 1652-1654.

34. Stoll BA. Western nutrition and the insulin resistance syndrome: a link to breast cancer. Eur J Clin Nutr 1999; 53: 83-87.

35. Clayton SJ, May FE, Westley BR. Insulin-like growth factors control the regulation of oestrogen and progesterone receptor expression by oestrogens. Mol Cell Endocrinol 1997; 128: 57-68.

36. Westley BR, Clayton SJ, Daws MR, Molloy CA, May FE. Interactions between the oestrogen and insulin-like growth factor signalling pathways in the control of breast epithelial cell proliferation. Biochem Soc Symp 1998; 63: 35-44.

37. Molloy CA, May FE, Westley BR. Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line. J Biol Chem 2000; 275: 12565-12571.

38. Ellis MJ, Jenkins S, Hanfelt J et al. Insulin-like growth factors in human breast cancer. Breast Cancer Res Treat 1998; 52: 175-184.

39. Su YA, Ray ME, Lin T et al. Reversion of monochromosome-mediated suppression of tumorigenicity in malignant melanoma by retroviral transduction. Cancer Res 1996; 56: 3186-3191.

40. Miki Y, Nishisho I, Horii A et al. Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. Cancer Res 1992; 52: 643-645.

41. Mitreiter K, Schmidt J, Luz A et al. Disruption of the murine p53 gene by insertion of an endogenous retrovirus-like element (ETn) in a cell line from radiation-induced osteosarcoma. Virology 1994; 200: 837-841.

42. van Lohuizen M, Berns A. Tumorigenesis by slow-transforming retroviruses--an update. Biochim Biophys Acta 1990; 1032: 213-235.

43. Peters G. Oncogenes at viral integration sites. Cell Growth Differ 1990; 1: 503-510.

44. Ben David Y, Bernstein A. Friend virus-induced erythroleukemia and the multistage nature of cancer. Cell 1991; 66: 831-834.

45. Hanna Z, Jankowski M, Tremblay P et al. The Vin-1 gene, identified by provirus insertional mutagenesis, is the cyclin D2. Oncogene 1993; 8: 1661-1666.

46. Habets GG, Scholtes EH, Zuydgeest D et al. Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell 1994; 77: 537-549.

47. Dorssers LC, van Agthoven T, Dekker A, van Agthoven TL, Kok EM. Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. Mol Endocrinol 1993; 7: 870-878.

48. Lu SJ, Man S, Bani MR et al. Retroviral insertional mutagenesis as a strategy for the identification of genes associated with cis-diamminedichloroplatinum(II) resistance. Cancer Res 1995; 55: 1139-1145.

49. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci U S A 1993; 90: 8392-8396.

50. Thony B, Leimbacher W, Stuhlmann H, Heizmann CW, Blau N. Retrovirus-mediated gene transfer of 6-pyruvoyl-tetrahydropterin synthase corrects tetrahydrobiopterin deficiency in fibroblasts from hyperphenylalaninemic patients. Hum Gene Ther 1996; 7: 1587-1593.

51. Markowitz D, Goff S, Bank A. Construction and use of a safe and efficient amphotropic packaging cell line. Virology 1988; 167: 400-406.

52. Sambrook J, Fritsch EF, Maniatis T. Isolation of High-molecular-weight DNA from mammalian cells. In: Sambrook J, Fritsch EF, Maniatis T (Eds.). *Molecular Cloning: A Laboratory Manual, 2nd edition.* Plainview, NY, Cold Spring Harbor Laboratory Press, 1989: pp. 9.14-9.19.

53. Sambrook J, Fritsch EF, Maniatis T. Extraction and purification of plasmid DNA. In: Sambrook J, Fritsch EF, Maniatis T (Eds.). *Molecular Cloning: A Laboratory Manual, 2nd edition.* Plainview, NY, Cold Spring Harbor Laboratory Press, 1989: pp. 1.21-1.28.

54. Su YA, Lee MM, Hutter CM, Meltzer PS. Characterization of a highly conserved gene (OS4) amplified with CDK4 in human sarcomas. Oncogene 1997; 15: 1289-1294.

55. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 215: 403-410.

56. Gish W, States DJ. Identification of protein coding regions by database similarity search. Nat Genet 1993; 3: 266-272.